Gating of mammalian cardiac gap junction channels by transjuncional voltage

Hong-Zhan Wang,* Jian Li, Larry F. Lemanski, and Richard D. Veenstra*
Departments of *Pharmacology and 3Anatomy and Cell Biology, State University of New York Health Science Center, Syracuse, New York 13210

ABSTRACT Numerous two-cell voltage-clamp studies have concluded that the electrical conductance of mammalian cardiac gap junctions is not modulated by the transjunctional voltage (Vj) profile, although gap junction channels between low conductance pairs of neonatal rat ventricular myocytes are reported to exhibit Vj-dependent behavior. In this study, the dependence of macroscopic gap junctional conductance (gij) on transjunctional voltage was quantitatively examined in paired 3-d neonatal hamster ventricular myocytes using the double whole-cell patch-clamp technique. Immunolocalization with a site-specific antiserum directed against amino acids 252–271 of rat connexin43, a 43-kD gap junction protein as predicted from its cDNA sequence, specifically stained zones of contact between cultured myocytes. Instantaneous current–voltage (I, Vj) relationships of neonatal hamster myocyte pairs were linear over the entire voltage range examined (0 ≤ Vj ≤ ±100 mV). However, the steady-state I, Vj relationship was nonlinear for Vj > ±50 mV. Both inactivation and recovery processes followed single exponential time courses (tactivation = 100–1,000 ms, t recovery = 300 ms). However, I, recovered rapidly upon polarity reversal. The normalized steady-state junctional conductance–voltage relationship (Gij, Vj) was a bell-shaped curve that could be adequately described by a two-state Boltzmann equation with a minimum Gij of 0.32–0.34, a half-inactivation voltage of –69 and +61 mV and an effective valence of 2.4–2.8. Recordings of gap junction channel currents (iij) yielded linear iij, Vj relationships with slope conductances of ~20–30 and 45–50 pS. A kinetic model, based on the Boltzmann relationship and the polarity reversal data, suggests that the opening (α) and closing (β) rate constants have nearly identical voltage sensitivities with a V1/2 of ±62 mV. The data presented in this study are not consistent with the contingent gating scheme (for two identical gates in series) proposed for other more Vj-dependent gap junctions and alternatively suggest that each gate responds to the applied Vj independently of the state (open or closed) of the other gate.

INTRODUCTION

Gap junctions mediate the electrical cell-to-cell communication necessary for synchronous impulse propagation and initiation of contraction in cardiac tissues (Page and Manjunath, 1986). Hexameric assemblies of integral membrane proteins form hemichannels, or connexons, which align with connexons of an adjacent cell to form patent channels (Makowski et al., 1977). It is now generally accepted that there is a family of structurally related gap junction proteins, or connexins, that contain both highly conserved and unique primary amino acid sequences (Beyer et al., 1990). The different connexins have partially overlapping tissue distributions. Molecular cloning techniques and site-directed antibodies have identified close homologies of connexin43 as the predominant gap junction protein in a variety of species of mammalian heart (Beyer et al., 1987, 1989; Yancey et al., 1989; El-Aoumari et al., 1990; Fishman et al., 1990). The best evidence that connexin43 forms junctional channels comes from the functional expression of this protein in paired Xenopus oocytes injected with rat connexin43 mRNA or in communication-deficient SKHeP1 cells transfected with human connexin43 cDNA (Swenson et al., 1989; Werner et al., 1989; Fishman et al., 1990). Injection of rat connexin43 mRNA into Xenopus oocytes results in the formation of large junctional conductances (gij) that are not sensitive to transjunctional potentials (Vj) of less than ±60 mV (Swenson et al., 1989; Werner et al., 1989). In these same studies, it was also observed that connexin43 is capable of forming hybrid channels with an endogenous Vj-sensitive connexin, presumably connexin38 (Ebihara et al., 1989). In paired cardiac myocytes from adult rat or guinea pig heart, gj was found to be insensitive to transjunctional potentials (Kameyama, 1983; Metzger and Weingart, 1985; White et al., 1985; Weingart, 1986; Noma and Tsuibo, 1987; Rüdisüli and Weingart, 1989). This is contrary to observations in embryonic chick heart (Veenstra, 1990; 1991a), but these functional differences between species may be conferred by the presence of different connexins (Beyer et al., 1990; Veenstra 1991a).

Recently, Rook et al. (1988, 1990) have observed that newly formed gap junction channels between neonatal rat ventricular myocytes behave in a voltage-dependent manner when transjunctional voltage (Vj) exceeds ±50 mV. This Vj-dependent behavior was observed provided that gj < 1 nS. Junctional currents attributable to the opening and closing of single gap channels have been detected in paired neonatal and adult mammalian cardiac myocytes with corresponding channel conductances of 32 or 50 pS (Burt and Spray, 1988; Rook et al., 1988, 1989; Rüdisüli and Weingart, 1989). These values agree closely with channel conductance values of 60 pS obtained in SKHeP1 cells stably transfected with human connexin43 cDNA (Fishman et al., 1990, 1991).

Address correspondence to Dr. Veenstra.

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This study was undertaken to determine the precise relationship between transjunctional voltage and junctional conductance in mammalian cardiac gap junctions containing connexin43. We found that gap junctions of neonatal hamster ventricular myocardium stained specifically with antisera raised against a unique rat connexin43 peptide sequence. Unlike previous reports, cell pairs with a junctional conductance of 12 nS still exhibited a marked voltage-dependent gating when the transjunctional voltage exceeded ±50 mV which could be described by a two-state Boltzmann distribution. Preliminary observations of these results have been presented elsewhere (Veenstra, 1991b; Wang et al., 1991).

MATERIALS AND METHODS

Cell isolation and culture

Syrian hamsters were obtained from our breeding colony maintained in the Central Animal Care Facility at the State University of New York, Health Science Center at Syracuse. To prepare the primary heart cell cultures, myocytes were isolated from heart ventricles of 3-d neonatal Syrian hamsters. The animals were killed by cervical dislocation and the hearts immediately removed using sterile techniques. The extirpated hearts were washed twice in cold Hank’s solution (Gibco Laboratories, Grand Island, NY) to remove residual blood. The ventricles were dissected free and minced into 1–2-mm³ pieces using a new scalpel blade under a dissecting microscope. The pieces were washed twice with ice-cold Hank’s solution, then treated with 0.08% trypsin and 0.01% collagenase in Hank’s solution for 10 min at 37°C in an incubator with gentle agitation. The first supernatant was discarded and the supernatant from the next three cycles was diluted twofold with cold culture medium composed of Earle’s MEM, 15% FCS, 200 mM glutamine, 100 U/ml penicillin, and 100 mg/ml streptomycin. Cells were harvested from the enzymatic solution by centrifugation, and the cell pellet was resuspended in fresh culture medium. The cell culture was enriched for myocytes by a differential adhesion step (Lemanski and Tu, 1983). The final density of cells was adjusted to ≅2 × 10⁵ dispersed cells/ml of medium. The cells were grown on gelatin-coated glass coverslips in 35-mm-diam plastic culture dishes, and incubated at 37°C in a humidified atmosphere of 5% CO₂ and 95% air. The culture medium was changed every 48 h.

Fixation and immunofluorescent staining

Cells grown on coverslips for 2–3 d were removed from the culture dishes and washed three times with PBS (pH 7.4) composed of 0.15 M NaCl and 10 mM phosphate buffer. Cells were fixed and permeabilized in cold acetone for 30 s at −20°C. After 2 min of air drying at 22°C, the coverslips were washed again in PBS three times for 3 min each, then stored at 4°C in excess PBS. Rabbit antiserum against rat connexin43 amino acid residues 252–271 (courtesy of Dr. Eric C. Breyer, Washington University, St. Louis, MO) was used for primary staining of gap junction protein. A complex of biotinylated donkey anti-rabbit IgG secondary antibody (Lot #62) and Texas Red-streptavidin (Amer sham Corp., Arlington Heights, IL) antibody, diluted in PBS, was used as a fluorescent marker for anti-connexin43 antibody.

After the coverslips with the attached cells were removed from the PBS, they were incubated for 15 min at 37°C in 3% filtered nonfat milk to block nonspecific staining. This was immediately followed by a 60-min incubation with anti-connexin43 diluted 1:40 with PBS. The cells were washed three times in PBS, blocked with 3% milk again, incubated with biotinylated anti-rabbit secondary antibody (1:20 dilution) in a humidified chamber at 37°C for 1 h or 4°C for overnight. The cells were then incubated with Texas Red-streptavidin at 37°C for 1 h or 4°C for overnight and washed with PBS. Cells were viewed with a light microscope (Universal: Carl Zeiss, Inc., Thornwood, NY) equipped with epifluorescent illumination using a mercury light source and photographed on Kodak plus-X film.

Junctional current and conductance measurements

All electrophysiology experiments were performed at room temperature according to previously published procedures (Veenstra and DeHaan, 1986; Veenstra, 1990). Plates of cultured myocytes were rinsed five to seven times with balanced salt solution (BSS, mM: 142 NaCl, 1.3 KCl, 0.8 MgSO₄, 0.9 NaH₂PO₄, 1.8 CaCl₂, 5.5 dextrose, and 10 HEPES, pH 7.2) and placed on the stage of an inverted phase contrast light microscope (IMT-2; Olympus Corp. of America, New Hyde Park, NY) for observation. Gf seals were formed on each cell of a pair using patch pipettes with tip resistances of 4–6 MΩ when filled with standard internal pipette solution (IPS, mM: 110 Kglutamate, 15 NaCl, 1 KH₂PO₄, 4.6 MgCl₂-6H₂O, 5 EGTA, 3 Na₂ATP, 3 Na₂phosphocreatine, and 25 HEPES). Access to the cell interior was gained by the application of a pulse of suction. Whole-cell capacitive currents in response to a 10-mV depolarizing step (holding potential = −80 mV) were simultaneously recorded using a 1 MHz A/D board (RC Electronics, Inc., Santa Barbara, CA). Electrode resistances following patch break were determined from the capacitive current transients of each cell as previously described (Veenstra and DeHaan, 1988). Settling time for the voltage clamp circuit was ≤800 μs. Whole-cell electrode resistances ranged from 14 to 34 MΩ (mean ± SEM: 20.1 ± 1.8 MΩ, n = 16). Cell input resistances ranged from 1 to 5 GΩ. Because series resistance was typically <3% of the cell input resistance, series resistance compensation was not used.

Junctional currents were measured directly under dual whole-cell patch-clamp conditions as previously described (Veenstra, 1990, 1991a). Briefly, a transjunctional voltage gradient (ΔV) is applied by setting the command potentials (V₁ and V₂) to different values and measuring the whole cell currents (I₁ and I₂) produced under these conditions. This was accomplished by initially setting V₁ - V₂ = −40 mV for 3 s to determine the membrane holding current for each cell, followed by the application of a 2-s hyperpolarizing or depolarizing voltage pulse to cell 1 (ΔV₁ = −V₁) while maintaining V₂ constant. During each ΔV₁ pulse, each patch-clamp amplifier will supply an additional whole-cell current (ΔI₁ and ΔI₂) to maintain V₁ and V₂ at their respective values. Junctional current (Iᵦ) will be of opposite sign in the two cells because junctional resistance (JR) is isolated from ground by the input resistance (JRₑ) of each cell (Veenstra and DeHaan, 1988). JRₑ is determined by the parallel combination of the seal resistance (JRₛ) and membrane resistance (JRₑ) of the cell. Any additional nonjunctional membrane currents will have the same sign in both cells because the direction of current flow is the same (from cell interior across JRₛ to ground) in this case. Only the change in I₂ (ΔI₂) during the ΔV₁ pulse is taken as a direct measure of Iᵦ, whereas ΔI₁ equals the negative of Iᵦ (relative to I₂) plus the capacitive and nonjunctional membrane currents (Iₑₑ) associated with the change in V₁. Junctional conductance (gᵦ) is calculated according to Ohm’s law, or gᵦ = Iᵦ/ΔV₁. The accuracy of this conventional gᵦ measurement depends on two major assumptions: that Vᵦ = −ΔV₁ and that ΔI₂ = ΔI₁. In actual terms, the membrane potential (Vm) for each cell differs from V by an amount equal to −Rᵦ × Iᵦ where Rᵦ is the resistance of the patch electrode. Furthermore, it can be shown that Iᵦ = ΔI₁ × (1 + Rᵦ/Rₑₑ) (Rook et al., 1988, Giaume, 1991). So gᵦ = [ΔI₁ × (1 + Rᵦ/Rₑₑ)]/|−ΔV₁ − (ΔI₁ × Rᵦ − ΔI₂ × Rₑₑ)|. The fraction of Iᵦ that is absent from ΔI₁ must flow across Rₑₑ to ground and therefore alter Vₑₑ. The resulting change in Vₑₑ can be expressed as ΔVₑₑ = Iᵦ × Rᵦ/Rₑₑ where Rₑₑ = (Rᵦ × Rₑₑ)/(Rᵦ + Rₑₑ). It can also be shown that ΔVₑₑ = gᵦ(ΔV₁/|1 + (Rᵦ/Rₑₑ)|) × ([(Rᵦ + Rₑₑ)/Rᵦ] × Rᵦ) where Rᵦ = 1/gᵦ and Vᵦ = ΔVₑₑ − ΔVᵦ. Hence, errors in estimating gᵦ will depend on the Rᵦ/Rₑₑ and Rᵦ/Rₑₑ. For the experiments presented in this study, Rᵦ/Rₑₑ ≤ 3%.
measurements of discrete quantal changes of Ij is observed from Ii is poorly defined because the time duration (area under the peak) amounted to <1% of the cumulative recording time. The difference between successive peaks corresponds to the mean single channel current (ij) for the observed activity. These values were 3.5 (n = 19 channel events), 2.6 (n = 129), 3.4 (n = 69), and 3.7 pA (n = 23), respectively (beginning with the first peak above baseline). (C) Junctional current record (ΔIj) from a second cell pair during a +80-mV Vj step beginning at the arrow indicating the direction of channel openings. The thin horizontal line indicates the ground state (Ij = 0 pA), and the lowercase letters (a and b) indicate breaks made in the continuous 22-s record for display purposes only. (D) Amplitude histogram for the channel activity shown in C. The arrows point to the threshold levels used for detecting channel transitions. The mean single channel currents were 1.9 and 1.3 pA.

Each peak corresponds to one of the six discrete levels indicated by the solid lines in Fig. 1 A. and the area under each peak is proportional to the time spent in each state (level). Single-channel currents (ij) are taken as the difference between the means of two adjacent peaks and the single-channel conductance (γj) can be calculated accordingly (γj = ij/Vj). Beginning with the first channel above baseline in Fig. 1 B, γj equals 50, 37, 49, and 52 pS, respectively.

In three experiments, resolution of channel currents of ±2 pA could be resolved. The channel activity shown in Fig. 1 C was obtained from a different cell pair under identical experimental conditions. The arrow indicates the direction of open-channel currents observed with the onset of a +80-mV Vj pulse, which remained on for the remainder of the 22-s continuous current record displayed in this panel. The amplitude histogram is shown in Fig. 1 D and the channel currents measured 1.9 and 1.3 pA, or γj = 24 and 16 pS. Channel events are counted by setting a threshold detector equal to the valley between adjacent peaks in the amplitude histogram and scanning the Ij trace a second time. Threshold levels for counting channel events are optimally >2 SD from the mean of each peak. Only events with a duration >5 points (=5 ms) are counted as an event. For the example shown here, 44 transitions...
were detected, omitting the initial step associated with the onset of the pulse. These procedures were followed for all experiments presented in this manuscript.

RESULTS

Immunohistochemical localization of gap junctions in vitro

After 1–2 d in vitro, cardiac myocytes appear round in shape (few cytoplasmic projections) compared to fibroblasts, which spread out more rapidly in culture (Li and Lemanski, 1990). To determine if 3-d neonatal hamster cardiac myocytes express connexin43 in vitro, cultured ventricular myocytes were stained with anti-connexin43 252–271 antiserum (Beyer et al., 1989) using standard immunohistochemical techniques. Fig. 2 displays a phase contrast micrograph and immunofluorescent image of such a cardiac myocyte cell pair. In both micrographs, the arrow indicates the location of intercellular contacts between the two cells. Punctate labeling of the intercellular surface with anti-connexin43 antiserum is evident in Fig. 2 B. Some cell clusters, presumably containing fibroblasts, did not stain with the connexin43 antiserum.

Regulation of junctional current by transjunctional potentials

To assess the relationship between transjunctional voltage and junctional current, $V_j$ was varied between the values of ±100 mV by systematically altering the membrane voltage of one cell ($V_{m1}$) from a common holding potential of −40 mV in 10-mV increments. Every 2-s duration $V_j$ pulse was separated by a 5-s recovery interval ($V_j = 0$ mV). Representative currents from one neonatal hamster cell pair are presented in Fig. 3 A. Instantaneous $I_j$, measured as the change in $I_j$ during the first 10 ms of each $V_j$ pulse, increases linearly with $V_j$ of either polarity. Beyond ±50 mV, $I_j$ undergoes a time- and $V_j$-dependent decay to new steady-state values. Both instantaneous and steady-state $I_j$ values are plotted as a function of $V_j$ in Fig. 3 B. The instantaneous $I_j$–$V_j$ relationship, illustrated by the open squares, was linear, with the slope of the line being equal to $g_j$ of the preparation (12.23 nS). Steady-state $I_j$, illustrated by the open diamonds in Fig. 3 B, was virtually identical to instantaneous $I_j$ in the −40 to +50-mV range, but deviates from linearity with further increases in $V_j$. Beyond ±70 mV, steady-state $I_j$ actually diminishes with increasing $V_j$, thus producing a negative slope conductance for the junctional membrane. Similar results were obtained from 11 other cell pairs where $g_j$ ranged from 0.82 to 13.29 nS (7.16 ± 1.30 nS, mean ± SEM). In three additional cell pairs, where $g_j$ of 36 to 40 nS were calculated from the slope of the linear instantaneous $I_j$–$V_j$ relationships (data not shown), steady-state $I_j$ had declined by <10% at $V_j$ of ±80 mV and steady-state $I_j$ was reduced by an average of only 15% from instantaneous $I_j$ values at $V_j$ = ±100 mV. In these three experiments, the measured $R_j$ of only 25–28 MΩ corresponds to the lower limit for the sum of $R_{dl1}$ and $R_{dl2}$. Under these conditions, $\Delta V_{m1}/\Delta V_j \approx 0.75$ and $I_j = 3.5–4.0$ nA at 100 mV results in a $\Delta V_{m2}$ of 40–50 mV. Hence, $V_j$ will equal only 25–35% of $\Delta V_j$ and very little time- and voltage-dependent decay would be expected to be observed under these circumstances.

The time course of the decline in $I_j$ was also examined at each $V_j$ above ±60 mV by fitting exponential functions to the decay phase of each $I_j$ trace. Accurate fits were obtained in eight experiments. Examples of the monoexponential fits obtained from one of these cell pairs are illustrated in Fig. 4. The corresponding $V_j$ is indicated to the left of each trace, and the smooth line represents the monoexponential fit of the data. There was close agreement between the theoretical curves and experimental data at all voltages examined regarding both the rate of decay and steady-state $I_j$. In some $I_j$ traces, a fast component ($r < 40$ ms) was also observed. Predicted steady-state $I_j$ values, determined by extrapolating the idealized fit to pulse durations of $r \rightarrow \infty$, varied by no more than 5% from the steady-state $I_j$ measured at the end of a 2-s pulse in 62 of 64 trials. The monoexponential time constants for all eight experiments are summarized in Table 1. There was a large amount of scatter from one experiment to the next, as indicated by the large SEM observed at $V_j$ < ±100 mV. Decay time constants decreased as $V_j$ increased in either direction, consistent with previous observations in other $V_j$-dependent cardiac gap junctions (Veenstra, 1990, 1991a).

Because $I_j$ undergoes a time-dependent decay at large potentials, experiments were performed to determine the rate of recovery from inactivating potentials. For these experiments, the recovery interval between inacti-
Vj

Ij

50

10

-100

-20

-40

-60

-80

100 pA

500 mSEC

(i) Instantaneous (inst) and steady-state (ss) Ij, for the experiment shown in Fig. 3A were plotted as a function of Vj. The instantaneous Ij-Vj relationship approximates a straight line with a slope of 12.23 nS. The steady-state Ij-Vj relationship deviates from linearity above ±50 mV and has a negative slope above ±70 mV. The lines connecting the data points were drawn by eye.

FIGURE 3 Voltage dependence of neonatal hamster cardiac gap junctions. (A) Functional current (Ij) during a 2-s pulse to the indicated transjunctional voltages (Vj) undergoes a time-dependent decay when Vj exceeds ±50 mV. Instantaneous (onset of pulse) junctional conductance (gj) values ranged from 10.64 to 12.80 nS and steady-state (end of pulse) gj values ranged from 4.49 to 12.20 nS for the current traces shown. (B) Instantaneous (inst) and steady-state (ss) Ij, for the experiment shown in Fig. 3A were plotted as a function of Vj. The instantaneous Ij-Vj relationship approximates a straight line with a slope of 12.23 nS. The steady-state Ij-Vj relationship deviates from linearity above ±50 mV and has a negative slope above ±70 mV. The lines connecting the data points were drawn by eye.
convenient method for normalizing the data from cell pairs having different $g_j$ values. The results of eight experiments are summarized in Fig. 7. Normalized $g_{sa}$ ($G_a$) ranged from 0.80 to 1.0 for $0 \leq V_j \leq \pm 50$ mV and declined to a minimum value of 0.32–0.34 at $ \pm 100$ mV. For each polarity, the $G_{sa}-V_j$ curve was fit by a Boltzmann equation of the form:

$$G_{sa} = \begin{pmatrix} (G_{max} - G_{min})/\left[1 + \exp(A(V_j - V_o))\right] + G_{min} \end{pmatrix}$$

where $G_{max}$ is the normalized maximum conductance (= 1), $G_{min}$ is the normalized minimum conductance for large transjunctional voltages, $V_o$ is the voltage where $G_{sa}$ lies halfway between $G_{max}$ and $G_{min}$, and $A$ is a parameter expressing the slope of the curve (Spray et al., 1981). The constant $A$ can be expressed as $zq/kT$, where $z$ is the valence of charge $q$ that act as the voltage sensor in the membrane to effect the transition from the open to closed conductance states, and $kT$ represent Boltzmann's constant and absolute temperature, respectively. For the negative polarities shown Fig. 7, $G_{min} = 0.34, A = -0.112$ (which corresponds to a valence of 2.8 electrons), and $V_o = -69$ mV. For the positive polarities, $G_{min} = 0.32, A = 0.093 (z = 2.4)$, and $V_o = +61$ mV.

**Behavior of gap junction channels**

In eight additional experiments, gap junction channel currents were resolved owing to the low $g_j$ of these cell pairs. Complete single gap junction channel current-voltage ($i_j-V_j$) relationships were determined in two of these eight experiments either by applying the voltage protocol described in Fig. 3 or by manually reproducing each $V_j$ step to increase the pulse duration (and the number of channel events). The results of these two experiments are illustrated in Fig. 8. Channel openings are clearly evident in Fig. 8, $A$ and $C$, as upward deflections in $i_j$ when $V_j$ is positive and downward deflections in $i_j$ when $V_j$ is reversed. As $V_j$ approaches 0 mV, $i_j$ decreases in amplitude and approaches the noise limit of the recordings. Thus, channel amplitudes were determined only in the range of $\pm 50$ to $\pm 100$ mV. Fig. 8 illustrates the channel activity observed during brief 2-s duration pulses to the indicated $V_j$. The amplitudes of the channel currents, determined using the procedures illustrated in Fig. 1 on 40–70-s continuous recording intervals, are plotted as individual points on the $V_j$ axis in Fig. 8 B. The solid line represents the best fit of the data as determined by the least-means-square criterion. Linear regression analysis produced identical results ($r = 0.96$). The straight line has a slope of 46 ± 2 pS, which is consistent with the single-channel conductance values ($\gamma_j$) reported for mammalian cardiac gap junction channels (Burt and Spray, 1988; Rook et al., 1988; Rüdisüli and Weigart, 1989). Similar results were obtained in the $\pm 80$ to $\pm 100$-mV range from four other cell pairs (Veentra, 1991b). Fig. 8, C and D, represents the gap junction...
channel activity and $i_j - V_j$ relationship obtained from a different cell pair using the same procedures. In this example, only 2-s-duration pulses were analyzed, so the event count is smaller than for the previous experiment. The slope of the linear $i_j - V_j$ relationship was $33 \pm 2 \text{ pS}$ ($r = 0.95$). Single channel conductances of 25–35 pS were observed in two other experiments when $-80 \leq V_j \leq \pm 100 \text{ mV}$. Many of the $i_j$ traces in Fig. 8, A and C, reveal the closure of one or more channels during the first 500 ms of the $V_j$ pulse, which indicates that the time-dependent decrease in $i_j$ may be due to the $V_j$-dependent closure of gap junction channels.

**Channel gating rate constants**

It is possible to calculate the channel opening and closing rate constants from the $G_{ss}$ values and first-order decay time constants presented in Figs. 4 and 7 and Table 1, assuming that the channel transitions between the open and closed states are due to the free energy difference (which varies as a function of transjunctional voltage) between the states (Harris et al., 1981). For the reaction scheme:

$$\text{open} \underset{\beta}{\rightleftarrows} \text{closed},$$

$\alpha$ and $\beta$ can be calculated from the decay time constant $\tau$ and the normalized steady-state $G_{ss}$ according to the following equations:

![Figure 5](image1.png)

**FIGURE 5** Time-dependent recovery of gap junctional conductance. The recovery interval between +80 mV $V_j$ pulses (2-s duration) was varied from 100 ms to 10 s in three cell pairs. For each experiment, instantaneous $g_j$ values were plotted as a function of recovery interval by normalizing $g_j$ to the value obtained with a 10-s recovery interval. Each point represents the mean $G_j$ (±SEM) and the solid line is a theoretical fit obtained using the equation $G_j = 1/[1 + \exp(-t/\tau)]$, where $\tau = 297 \text{ ms}$. All data points between 3 and 10 s were within ±1 SEM of the normalized maximum $G_j (=1)$.

![Figure 6](image2.png)

**FIGURE 6** Transient recovery of $I_j$ from inactivation upon polarity reversal. The control +80-mV $V_j$ pulse (top panel, first trace) was preceded by a time interval where both cells were clamped to a common holding potential of −40 mV while the test +80-mV $V_j$ pulse (top panel, second trace) was preceded by 2-s prepulse to $V_j = −80 \text{ mV}$. The decay phases of the same two traces are superimposed in the bottom panel for comparison. For the control pulse, instantaneous $I_j$ was 350 pA ($g_{ss} = 4.38 \text{ nS}$) and decayed to a steady-state $I_j$ of 126 pA ($g_{ss} = 1.58 \text{ nS}$) with a time constant ($\tau$) of 335 ms. After inactivation at −80 mV (bottom panel), instantaneous and steady-state $I_j$ still peaked at 284 and 113 pA ($g_{ss} = 3.55 \text{ nS}$ and $g_{ss} = 1.41 \text{ nS}$) and $\tau = 294 \text{ ms}$. Note that $I_j$ during the test pulse does not overshoot the control pulse.
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FIGURE 7  Steady-state junctional conductance–voltage relationship. Ratio of steady-state (ss) to instantaneous (inst) $g_j$ taken from eight different cell pairs. Each point represents the mean ± SEM. $G_{ss}$ declines with increasing $V_j$ in either direction with the greatest decrease in $G_{ss}$ occurring when $V_j$ exceeds ±50 mV. The solid line is a theoretical fit of the data assuming a two-state Boltzmann distribution (see text) with $G_{min} = 0.34$ and 0.32, $A = -0.112$ and 0.093, and $V_o = -69$ and +61 mV for negative and positive $V_j$, respectively.

\[
\alpha = G_s/\tau
\]

and
\[
\beta = (1 - G_s)/\tau,
\]

where $G_s = G_{ss} - G_{min}$. Using these equations, $\alpha$ and $\beta$ were calculated from the mean $G_{ss}$ and $\tau$ values presented in Fig. 7 and Table 1. The closing rate constant ($\beta$) was observed to be higher than $\alpha$ at large $V_j$ values ($V_j > V_o$). This is similar to the findings reported for other $V_j$-dependent gap junctions (Harris et al., 1981). The solid lines in Fig. 9 represent the best fit of the data according to the equations:

\[
\alpha = \lambda \exp[A_o(V - V_o)]
\]

and
\[
\beta = \lambda \exp[A_p(V - V_o)],
\]

where $\lambda$ is a constant equal to the rate when $\alpha = \beta$, which occurs at $V = V_o$. $A_o$ and $A_p$ are the respective voltage sensitivities of the opening and closing rates. For the curves shown in Fig. 9, $\lambda = 0.69$ and 0.67, $A_o = -0.06$ and 0.04, $A_p = -0.06$ and 0.07, and $V_o = -63$ and +62 mV for the negative and positive voltages, respectively. $\tau$ was calculated from Eqs. 4 and 5 as $1/(\alpha + \beta)$ and produced predicted decay constants of (mV, ms): -100, 148; -90, 262; -80, 437; -70, 635; -60, 739; 60, 750; 70, 628; 80, 381; 90, 200; 100, 100. These values agree closely with the values presented in Table 1 except for ±60 mV. $G_{ss}$ was also calculated using the equation $G_{ss} = (G_{max} - G_{min}) \times (\alpha/(\alpha + \beta)) + G_{min}$ and produced a curve nearly identical to the Boltzmann curve in Fig. 7 (data not shown). The term $[\alpha/(\alpha + \beta)]$ is equivalent to the steady-state open probability for the $V_j$-sensitive conductance.

DISCUSSION

Unitary channel conductance

The slope conductances of linear single gap junction channel current-voltage relationships provide reliable estimates of the mean gap junction channel conductances observed in low $g_j$ cell pairs. In paired neonatal hamster ventricular myocytes, we observed linear slope conductances of $33 \pm 2$ and $46 \pm 2$ pS (Fig. 8). Similar gap junction channel conductance ($\gamma_j$) values of 20–30 and 39–60 pS have been obtained from neonatal rat and adult guinea pig myocyte pairs (Rook et al., 1988, 1989; Burt and Spray, 1988; Rüdissl and Weingart, 1989) and immunocytochemical studies have revealed the presence of connexin43 in the junctional membranes of all adult mammalian ventricular myocytes in situ (Beyer et al., 1989; Yancey et al., 1989; El Aoumari et al., 1990) and in paired neonatal mammalian myocytes in vitro (Fig. 2; Laird and Revel, 1990). Based on the numerous channel conductance measurements from these preparations, it is generally agreed that a 40–60-pS channel is formed by connexin43. This concurs with the observation of a 60-pS channel following the transfection of SKHep1 cells with human connexin43 cDNA (Fishman et al., 1990, 1991).

In neonatal rat, a 30-pS channel conductance was attributed to the formation of heterologous fibroblast-myocyte gap junctions (Rook et al., 1989). Connexin43 is known to form heterologous gap junctions with connexin32 and connexin38 when expressed in Xenopus oocytes (Swenson et al., 1989; Werner et al., 1989). The formation of heterologous gap junctions could explain our observation of a 30-pS channel in three cell pairs (Fig. 8, C and D), although we cannot definitively confirm the identity of a fibroblast in these cases. Based on the observations of contractile activity and the extent of cell spreading during the first 24 h in culture, we estimate that >80% of the cells we recorded from were myocytes. We also cannot rule out the possibility that a 30-pS channel is due to the presence of other connexins, as observed in embryonic chick heart and liver hepatocytes (Zhang and Nicholson, 1989; Beyer et al., 1990), or that it is a subconductance state of a 60-pS channel. However, identification of a subconductance state requires the observation of the substate only in the presence of the larger channel (Fox, 1987), which was not observed for the experiment presented in Fig. 8, C and D. Recently, Kanter et al. (1992) have demonstrated the presence of mammalian homologs to chick connexin42 and connexin45 (named dog connexin40 and connexin45) in canine ventricular myocytes at the protein level. Chick connexin45 reportedly forms a 30-pS channel (Veenstra et al., 1992). In SKHep1 cells, low levels of endogenous coupling were manifested by the presence of a 30-pS channel, which was not identified by antibodies to con-
nexins26, -32, or -43 (Eghbali et al., 1990). The identification and functional expression of individual cardiac myocyte and fibroblast connexins by stable transfection in mammalian cell lines should provide the best method for determining the conductance properties of the connexin proteins.

**Effects of transjunctival potential on junctional currents**

In this investigation, the $V_j$ dependence of mammalian cardiac gap junctions between paired 3-d neonatal hamster ventricular myocytes was quantitatively assessed. The results indicated a symmetrical drop in $G_{sh}$ above $V_j$ values of ±50 mV and are suggestive of a bilateral voltage-gated mechanism. The time-dependent decay and recovery processes followed first-order kinetics with time constants of similar dimensions. These observations are contrary to earlier studies in paired adult cardiac myocytes, although one explanation for the observed differences is that $V_j$ did not exceed ±50 mV in many of the previous experiments (Kameyama, 1983; White et al., 1985; Weingart, 1986). A second explanation for the apparent lack of voltage dependence is that the large $g_j$ of adult myocyte preparations precludes the observation of $V_j$-dependent behavior due to reductions in the actual applied $V_j$ gradient, as was observed in three experiments in this study where calculated $g_j > 36$ nS. This occurs as the junctional resistance approaches the values of the electrode and cytoplasmic access resistances, thereby reducing the transjunctival voltage sensed by the gap junction channels. In the neonatal rat, Rook et al. (1988) did observe $V_j$-dependent behavior when $g_j < 1$ nS and $V_j > ±50$ mV, but $V_j$ sensitivity was not observed when $g_j$ exceeded 1 nS in the same cell pair. They attributed this phenomenon to an increasing cytoplasmic voltage drop as gap junction plaques increase in size (Jongsma et al., 1991). Our findings indicate that the $V_j$ sensitivity was still readily observed in cell pairs with $G_j$ of 12 nS (Fig. 3), whereas a gradual loss of $V_j$ sensitivity occurred somewhere between 12 and 36 nS. One likely explanation for the observed difference in these two neonatal preparations is that the access resistances of the

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**FIGURE 8** Single gap junction channel current–voltage relationships. (A) Gap junction channel activity recorded during 2-s pulses to the indicated $V_j$. Analog low pass filter frequency = 125 Hz and digital sample rate = 1 kHz. (B) Each point represents the current amplitude as determined from a current amplitude histogram compiled from 40–70 s of recording time. Number of channel events at each $V_j$ ranged from 38 to 440. The solid line is a linear fit of the data with a slope of 46 ± 2 pS. (C) Gap junction channel activity from a different cell pair under identical experimental conditions. (D) Each point represents the current amplitude as determined from the current amplitude histogram compiled from each 2-s pulse. Number of observed channel events at each $V_j$ ranged from 5 to 19. The solid line is a linear fit of the data with a slope of 33 ± 2 pS.
Each peak in a channel amplitude histogram) with increasing \( V_j \), as observed in one neonatal hamster cell pair, are suggestive of such a mechanism (Veenstra, 1991 c). Furthermore, ensemble averaging of 5–10 gap junction channel current traces produces an exponential decay in \( I_j \) similar to observed macroscopic currents from higher conductance gap junctions of similar origin (Rook et al., 1988; Veenstra, 1990, 1991a). Based on the observed \( V_j \)-dependent behavior, Rook et al. (1988) predicted the channel open-state probability \( (P_o) \) to vary from \( \sim 0.25 \) at \( V_j = \pm 100 \) mV to near 0.85 at \( \pm 50 \) mV. From the Boltzmann distribution presented in Fig. 7, \( G_{ss} \) equals 0.84–0.93 at \( V_j = \pm 50 \) and 0.32–0.34 at \( \pm 100 \) mV. Because \( P_o = \alpha / (\alpha + \beta) \) in the steady state, we can obtain estimates of \( P_o \) at each voltage from the kinetic analysis presented in Fig. 9. Our calculations predict a maximum \( P_o \) of 1.0 at \( V_j = 0 \) mV and \( \sim 0.80 \) at \( V_j = \pm 50 \) and a minimum \( P_o \) near zero at \( V_j = \pm 100 \) mV. These estimates were based on the presence of only a single population of channels with simple two-state gating kinetics. Of course, this is only a \( P_o(V_j) \) term, which applies to the already open channel, so the actual \( P_o \) for all gap junction channels could still be much less than 1.0 at low \( V_j \) values because other factors may cause some gap junction channels to be closed at rest \( (V_j = 0 \) mV). This interpretation is slightly different from previous correlative electrophysiological and morphological studies in neonatal rat heart, where it was suggested that virtually all channels are open at rest (Rook et al., 1990).

**Kinetic considerations**

Calculation of the theoretical time constants according to the relation \( \tau = 1 / (\alpha + \beta) \), where \( \alpha \) and \( \beta \) are determined by the Boltzmann relations presented in Eqs. 4 and 5, produced values of \( \sim 100–150, \) 200–260, \( 380–440, \) 620–640, and \( 740–750 \) ms for \( V_j = \pm 100, \) \( \pm 90, \) \( \pm 80, \) \( \pm 70, \) and \( \pm 60 \) mV, respectively. These values are in reasonable agreement with the experimental values presented in Table 1, as are the experimental and theoretical rate constants presented in Fig. 9, with the possible exception of \( \pm 60 \) mV. The discrepancies between the experimental and theoretical values at \( \pm 60 \) mV may result from the difficulty in obtaining an accurate fit of decay constants >700 ms from 2-s-duration pulses. Overall, the two-state Boltzmann model, originally described for \( V_j \)-dependent gap junctions between amphibian blastomers (Harris et al., 1981; Spray et al., 1981), provides an accurate description of the equilibrium and kinetic properties of \( V_j \)-dependent mammalian cardiac gap junctions. In contrast to the findings in amphibian blastomers, the slope factors \( A_1 \) and \( A_4 \) are nearly identical. Because \( \alpha > \beta \) in any first-order system when \( V_j < V_o \) and vice versa, \( P_o \) is higher at low \( V_j \) values. In cardiac gap junctions, \( \beta \) is twice \( \alpha \) at 100 mV, whereas \( \alpha \) is 10 times greater than \( \beta \) at 0 mV.

The transient recovery of \( I_j \) after polarity reversal re-
veals further information about the gating behavior of the gap junction channels. Alternative models for the gating behavior of two gates in series with each other were advanced by Harris et al. (1981). The contingent and independent gating models both account for a transient rise in $I_g$ upon polarity reversal with one major difference. The contingent gating model requires that the closed gate of the polarized channel must open before the other gate in series can respond to the transjunctional voltage. This hypothesis is schematically represented as

$$C_{1O_2} \frac{m}{\alpha_1} O_{2} \frac{m}{\alpha_2} O_2 C_2.$$

With the contingent gating model, overshooting junctional currents were produced during a given transjunctional voltage pulse when preceded by prepulses of opposite polarity. According to the independent gating model, junctional currents elicited during a $V_g$ pulse preceded by a prepulse of opposite polarity do not overshoot junctional currents elicited by the same $V_g$ pulse in the absence of a prepulse. This model assumes that the gating of each gate by the applied voltage field occurs independently of whether the other gate is open or closed (Harris et al., 1981). In the experiment presented in Fig. 6, we observed little difference between control (no prepulse) and $-80$ mV prepulse test responses in a total of five experiments. These results appear to contrast with the findings of a contingent gating scheme for voltage-dependent gap junctions of amphibian blastomeres.

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